BIOTRANSFORMATION AND PROTEIN BINDING OF CANNABINOIDS

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1975

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Papers discussed in this survey

In this survey the following papers, referred to by the Roman numerals, will be discussed. Some additional and preliminary results are also presented.

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J. Pharm. Pharmacol. 25, 453 (1973)

X M. Widman, S. Agurell, M. Ehrnebo and G. Jones.
Binding of (+)- and (-)-Δ^1-tetrahydrocannabinols and (-)-7-hydroxy-Δ^1-tetrahydrocannabinol to blood cells and plasma proteins in man.
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Introduction

Cannabis sativa L., the hemp plant from which marihuana and hashish are derived, has a long history of use as a source of fiber as well as a drug. The first recorded use of cannabis as a remedy occurred in China nearly 5000 years ago. In recent years the intoxication with cannabis has increased rapidly among young people in Europe and in the U.S. This development has generated a debate about the possible harmful effects of the drug.

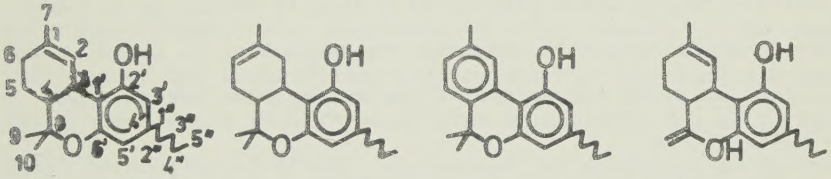
Before 1965, the intoxicating effect of cannabis could not be related to a specific compound. Since the materials used were not fully defined, most of the biological and clinical data published until then must be treated cautiously. It is only during the past few years that the complex chemistry of cannabis has been clarified and comprehensively reviewed [1,2].

Mechoulam and Gaoni [3-6] were the first to isolate the major psychoactive compound of cannabis in a pure form and assigned it the structure of (-)-3,4-*trans*-Δ^1-tetrahydrocannabinol (Δ^1-THC; 1; Fig. 1).

The isomer (-)-Δ^6-THC (2; Fig. 1), usually present in small amounts in the plant [7,8] has an activity similar to that of Δ^1-THC [9-11].

The effect of THC in man has been demonstrated to be similar to the effects of the plant material [12]. Initially, there is a period of stimulation with anxiety, heightened perceptions and euphoria, followed by a period of sedation and sleepiness. With higher doses hallucinogenic effects are observed [13]. So far very little is known about the mechanism by which THC alters the mental functions. The pharmacology of cannabis in humans and animals has recently been reviewed by Paton and Pertwee [14].

There are numerous other cannabinoids [6] present in cannabis of which cannabinol (CBN; 3, Fig. 1) and cannabidiol (CBD; 4, Fig. 1) are major compounds. CBN was isolated by Wood *et al.* in 1896 [15]and its structure was largely established by Cahn [16]. After synthesis of different CBN isomers, the correct structure of CBN was elucidated in 1940 [17,18]. CBN may or may not be present in fresh plant material, although during storage it becomes a major cannabinoid by oxidation of Δ^1-THC [6,19,20]. Adams [21] and Jacobs *et al.* [22] isolated CBD from plant material and suggested a structure which was not conclusively established until 1963 by Mechoulam and Shvo [23]. Both CBN and CBD are essentially psychoinactive *per se* [24,25], but there is evidence that they contribute to the overall effects of cannabis.



Δ^1-THC 1 (Δ^9-THC) Δ^6-THC 2 (Δ^8-THC) CBN 3 CBD 4

Fig. 1. Naturally occurring cannabinoids discussed in this survey. The monoterpenoid (Δ^1-THC) system of nomenclature is used throughout this discussion; the dibenzopyran (Δ^9-THC) system is given in parentheses.

The pharmacokinetics of cannabinoids in animals and humans could not be adequately studied prior to the synthesis of radiolabelled compounds. The preparation of radiolabelled tetrahydrocannabinols has recently been reviewed by Burstein [26]. After radiolabelled Δ^1-THC was synthesized, feasible distribution and elimination studies were carried out in animals and man [*cf.* 26,27]. In general, it was

found that Δ^1-THC was rapidly taken up into the tissues and rapidly converted to more polar compounds which were slowly excreted. High concentration of radioactivity was observed in the lung and the liver of rat and rabbit after i.v. administration or inhalation of radiolabelled Δ^1-THC [28-30]. In contrast, brain showed the lowest concentration of radioactivity of any tissue examined. Since THC exerts its effect predominantly on the central nervous system, these findings suggested it to be an extremely potent compound.

Scope of the present investigation

When this study was initiated, it was known that Δ^1-THC was extensively metabolized in the body of different species. However, no metabolites had been identified. Often biological activity and/or toxic effects of a drug is wholly or in part due to their metabolic products. Consequently, we found it important to study the nature of the metabolites formed from Δ^1-THC.

In papers I-III the *in vitro* metabolism of Δ^1-THC by the liver is described. Although the liver is considered to be the main site of drug metabolism, other tissues such as the lun may participate. This is shown in paper II which deals with the *in vitro* metabolism of Δ^1-THC by the lung. Since several isolated metabolites of Δ^1-THC have shown pharmacological activity, it has been suggested that the activity of Δ^1-THC is due wholly or in part at least, to its metabolites. Therefore, we isolated and identified brain metabolites of Δ^1-THC from the mouse and discussed the contribution of the metabolites to the pharmacological activity of Δ^1-THC (paper III). Bile metabolites of Δ^1-THC from the rat is characterized in paper IV

Since CBN and CBD are major cannabinoids and might contri-

bute to the overall effects of cannabis, their metabolism has likewise been of interest. In papers V and VI the *in vitro* metabolism of CBN by the liver of rat and rabbit is described. The *in vitro* metabolism of CBD by the liver of rat is treated in paper VII.

Distribution studies on radiolabelled THC indicated that the compound might be bound to proteins. This initiated our studies on the binding of Δ^1-THC and 7-hydroxy-Δ^1-THC to human plasma proteins (papers VIII and IX). The binding of Δ^1-THC and 7-hydroxy-Δ^1-THC to whole blood is described in paper X.

Biotransformation of Δ^1-THC

After i.v. administration of Δ^1-THC to animals it has been shown to be extensively transformed to several metabolites, some of which may be active. Since Δ^1-THC is a highly potent compound, only small doses can be administered which results in low concentrations of the metabolites. Difficulties in isolating and identifying these small amounts of *in vivo* metabolites led us to the *in vitro* metabolism of Δ^1-THC. We focused our attention on the monooxygenated metabolites *in vitro* since they may possibly occur *in vivo* as pharmacologically active compounds.

In vitro metabolism of Δ^1-THC by the liver

High concentration of radioactivity in the liver had been found in animals after i.v. administration of radiolabelled Δ^1-THC [28]. From this fact and the nature of THC, being a highly lipophilic drug, it could be expected to be a substrate for the hepatic microsomal enzyme system [31]. Paper I describes the incubation of (-)-Δ^1-THC-^{14}C with 10,000g supernatant obtained from rabbit liver with appropriate cofactors added [32]. After incubation, 40% of the added THC had been transformed to more polar compounds. One major metabolite (75% of the converted material) was isolated in a pure form.

The mass spectra (MS) revealed the molecular weight to be 330, consistent with the introduction of an oxygen into Δ^1-THC. The nuclear magnetic resonance (NMR) spectrum was largely in agreement with that of Δ^1-THC, but the signal at δ=1.69 ppm due to the vinylic methyl group in position 1 of Δ^1-THC was absent. Instead, a two proton singlet at δ=4.02 ppm was present, indicating that the hydroxylation had occurred at position 7.

Burstein *et al.* [33] reported the isolation and the identification of a metabolite of the isomeric Δ^6-THC obtained from rabbit urine. The structure of the metabolite was most likely that of 7-hydroxy-Δ^6-THC. Their metabolite was converted by p-toluene sulfonic acid to CBN which showed that the carbon skeleton was intact during the metabolism. Using the same method we converted the metabolite of Δ^1-THC to CBN.

The data presented above are consistent with the structure 9 (Fig. 2) for the isolated metabolite of Δ^1-THC.

Simultanously and independently, Wall *et al.* [34] reported the structure of 7-hydroxy-Δ^1-THC (9) from rat liver. Similarly, Burstein *et al.* [33], Ben-Zvi *et al.*[35] and Foltz *et al.*[36] established the structure of the corresponding 7-hydroxy-Δ^6-THC (21).

Further microsomal metabolites of Δ^1-THC (see Fig. 2) converted by rat liver are 6α,7-dihydroxy-Δ^1-THC (7) [34,37] 6α-hydroxy-Δ^1-THC (5) [37] and 7-oxo-Δ^1-THC (13) [38], the latter was isolated in a small yield. Using rat liver microsomes Δ^6-THC was converted to 7-hydroxy-Δ^6-THC (21) and also to a minor degree to 5α,7-dihydroxy-Δ^6-THC (16) and 5β,7-dihydroxy-Δ^6-THC (18) [39].

6β-hydroxy-Δ^1-THC (6) and 6α,7-dihydroxy-Δ^1-THC (7) have both been isolated from rabbit liver preparations [39]. Recently, Ben-Zvi *et al.* [40]found under similar conditions the 6α-isomer (5) together with 1,2α-epoxy-Δ^1-THC (10).

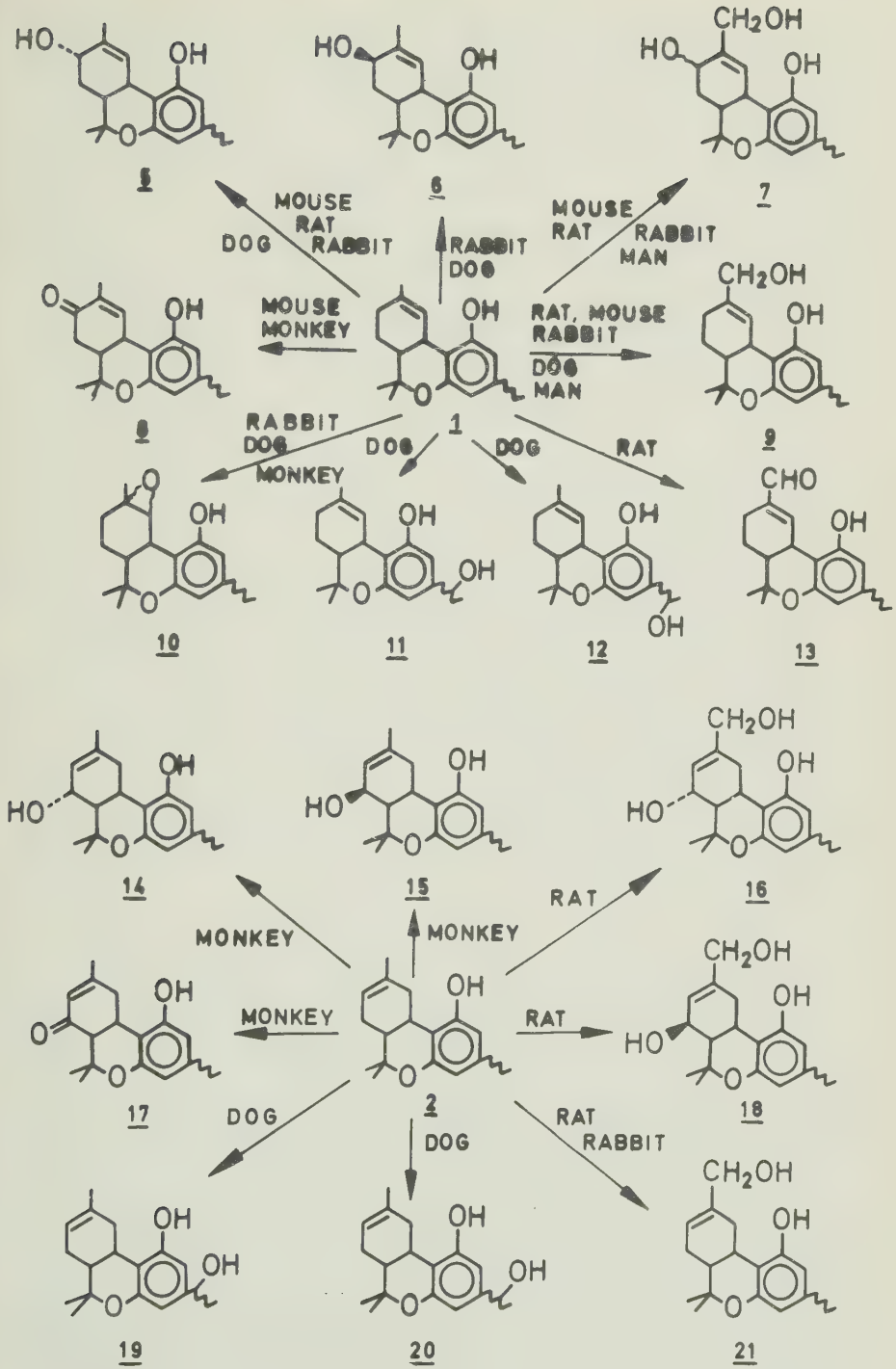


Fig. 2 Metabolic pathways for Δ^1-THC (1) and Δ^6-THC (2) using liver incubations.

The metabolism of Δ^1-THC in mouse liver has been studied simultanously and independently by Ben-Zvi *et al.* [37] and by us [paper III]. Both groups established by MS as well as GLC and TLC comparisons with authentic samples 7-hydroxy-Δ^1-THC (9) and 6α-hydroxy-Δ^1-THC (5) to be major metabolites in a hepatic mouse microsomal system. The NMR spectrum was also obtained by us for 6α-hydroxy-Δ^1-THC, which was in agreement with published data [35,41]. 7-hydroxy-Δ^1-THC together with 6,7-dihydroxy-Δ^1-THC have earlier been tentatively identified in mice and man by Christensen *et al.* [42]. In addition we isolated Δ^1-THC-6-one (8) which was formed in 1/12 of the yield of 7-hydroxy-Δ^1-THC. The isolated metabolite was identified by MS, GLC and TLC comparison with an authentic sample.

The metabolite Δ^1-THC-6-one has also been isolated from a liver microsomal fraction of squirrel monkey incubated with Δ^1-THC [43]. This investigation resulted in another unique metabolite of Δ^1-THC, namely 1,2-epoxy-hexahydrocannabinol (10). The epoxy group has been shown by Mechoulam *et al.* [44] to be in the α-position. Corresponding studies on Δ^6-THC resulted in the isolation of Δ^6-THC-5-one (17), 5α-hydroxy-Δ^6-THC (14) and 5β--hydroxy-Δ^6-THC (15).

The hydroxylations described above occur in positions allylic to the double bond in the terpene moiety. In paper II we are reporting two side-chain hydroxylated compounds of Δ^1-THC. When Δ^1-THC-^{3}H was incubated with a 10,000g supernatant of dog (greyhound) liver, several metabolites were isolated and identified (See also p.15). The major metabolites were assigned the structures of 6β-hydroxy-Δ^1-THC (6) and 6α-hydroxy-Δ^1-THC (5) by comparisons with MS, GLC and TLC with authentic samples. 7-Hydroxy-Δ^1-THC (9) and 1,2-epoxy-hexahydrocannabinol (10), which were both isolated in about 1/4 of the yield of 6β-hydroxy-Δ^1-THC,

were likewise identified by comparisons with synthetic reference compounds. The two side-chain hydroxylated compounds with the hydroxylations in positions 3" (11) and 4" (12) of the pentyl side-chain were both formed in about 1/8 of the yield of 6β-hydroxy-Δ^1-THC. The identification of these compounds will be discussed later in this survey (p.14). Maynard *et al.* [45] showed that Δ^6-THC could be hydroxylated in the pentyl side-chain to 1"-hydroxy-Δ^6-THC (19) and 3"-hydroxy-Δ^6-THC (20) by dog (beagle) liver supernatant.

In vitro metabolism of Δ^1-THC by the lung

Although the liver is considered to be the main site for THC metabolism, other tissues such as the lung, spleen and blood have been reported [42,46] to be metabolically active. Nakazawa and Costa [46] found several transformation products of Δ^1-THC when using lung microsomes from rat. They suggested 7-hydroxy-Δ^1-THC to be a lung metabolite after TLC comparison with a synthetic reference. Another metabolite found showed a TLC mobility between Δ^1-THC and 7-hydroxy-Δ^1-THC. Ben-Zvi *et al.* [37] have proposed it to be 6α-hydroxy-Δ^1-THC, since they have isolated a similar substance using mouse and rat hepatic microsomes.

Little has been reported on the fate of Δ^1-THC in lung tissue although accumulation of the drug has been found in this organ after inhalation [30] which is the usual way of cannabis administration. So far, no THC metabolite formed by the lung has been satisfactorily identified. In paper II we describe the metabolism of Δ^1-THC by the isolated perfused dog lung.

Δ^1-THC-^{3}H (9mg) was administered intravascularly in 70% aqueous ethanol. After 2 h of perfusion small amounts of several metabolites could be isolated and identified by MS. In Fig. 3 the elution pattern of the radioactivity extracted form the circuit

plasma is shown. The plasma and the lung tissue were found to contain the same metabolites. Fraction 1 (Fig. 3) was revealed by TLC to contain two metabolites.

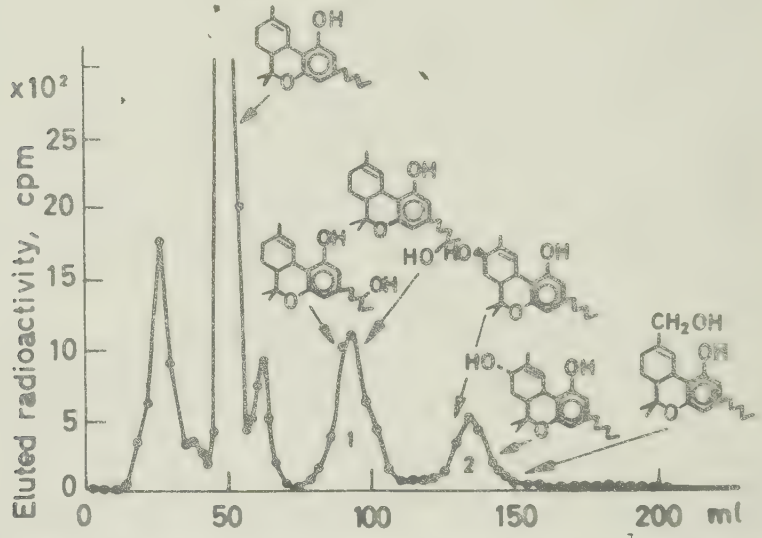


Fig. 3 Elution pattern on Sephadex LH-20 of metabolites of Δ^1-THC extracted from lung circuit plasma.

By MS both compounds showed the mass ion 330 and the fragment m/e 247 which is consistent with the introduction of a hydroxyl group in the side-chain of Δ^1-THC [47]. Comparisons by MS, GLC and TLC with synthetic 1"-,2"-,3"- and 5"-hydroxy-Δ^6-THC showed one of the metabolites to have properties similar to 3"--hydroxy-Δ^6-THC. From the mass spectra of the trimethylsilyl ether derivative the position of the hydroxyl group could be determined [*cf.* 48]. Thus, the structure of this metabolite was confirmed to be 3"-hydroxy-Δ^1-THC. The second metabolite showed after silylation the fragment m/e 117 indicating an α-cleavage between C-3" and C-4". Based on this and additional information it was determined to be 4"-hydroxy-Δ^1-THC.

After purification 3"-hydroxy-Δ^1-THC accounted for 14% and 4"-hydroxy-Δ^1-THC for 9% of the converted material. In addition

to the side-chain hydroxylated metabolites 7-hydroxy-Δ^1-THC, 6ß-hydroxy-Δ^1-THC and 6α-hydroxy-Δ^1-THC were isolated (fraction 2; Fig. 3) and identified by MS, GLC and TLC comparisons with synthetic references.

The same metabolic products were formed by the dog liver, using 10,000g supernatant. In addition, 1,2-epoxy-hexahydrocannabinol was isolated from the *in vitro* liver experiment. The metabolites were formed in different ratios by the two organs. 6ß-Hydroxy-Δ^1-THC together with 6α-hydroxy-Δ^1-THC were major liver metabolites but were isolated as minor compounds in the lung. Part of the difference in metabolism might be due to the experimental conditions with the use of an intact lung and a liver microsomal fraction. Although Nakazawa and Costa [46] used homogenates of both liver and lung of rat, they observed a qualitative difference between the two organs.

The synthetic 2"-,3"- and 5"-hydroxy-Δ^6-THC have been shown to be as active as Δ^6-THC in rhesus monkeys [2]. From this we can assume 3"-hydroxy-Δ^1-THC and 4"-hydroxy-Δ^1-THC to be pharmacologically active and thus be of importance for the overall effect of cannabis.

Metabolites of Δ^1-THC in brain

Several of the mono-oxygenated metabolites of Δ^1-THC so far isolated have shown pharmacological activity in animals and in humans. The relationships between structure and activity of the cannabinoids have been reviewed by Mechoulam *et al.* [2,49].

To assess the psychotropic effect of THC and its metabolites tests like dog ataxia, the Gayer corneal areflexia and the cataleptoid reactions in mice and rats have frequently been used [49]. Grunfeld and Edery [9] have suggested the rhesus monkey to be the most suitable model from experience with several animal species.

It has been suggested that the CNS activity of Δ^1-THC *in vivo* is mediated wholly or in part by its metabolites [*e.g.* 6,9,50]. To support such a hypothesis one has to know the metabolites in the brain and their relative potencies. When our study (paper III) of the brain metabolites was initiated, all previous identification of brain metabolites of Δ^1-THC in mice, rats and monkeys [*e.g.*51-53] had relied exclusively on the use of TLC. Our identification method was based on mass fragmentography together with other evidence.

Nineteen male albino mice were injected via a tail vein with Δ^1-THC-^{3}H (10 mg/kg). At maximal cataleptic activity (20min) the mice were killed, and the pooled brains were extracted. The purification on Sephadex LH-20 is shown in Fig. 4a. Fraction A had an elution volume of 34 ml and contained unchanged Δ^1-THC as indicated by mass fragmentography and TLC. A second fraction (B) with an elution volume of 80-150 ml was isolated. The combined fraction B (3 experiments), rechromatographed on Sephadex LH-20 (Fig. 4b), consisted of 9.3 µg of cannabinoids.

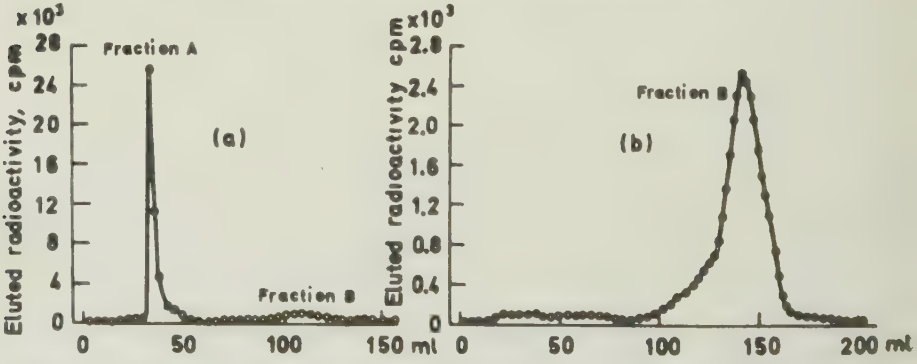


Fig. 4 Purification on Sephadex LH-20 of cannabinoids extracted from mouse brain 20 min after i.v. injection of Δ^1-THC-^{3}H (10 mg/kg).

This fraction was resolved into at least three components by TLC and into two compounds by mass fragmentography using m/e 271, 299, 312, and 330. The two major compounds of fraction B, accounting

for a total of about 70% of the radioactivity in the fraction, were identified as 6α-hydroxy-Δ^1-THC (27%) and 7-hydroxy-Δ^1-THC (43%). A minor component on TLC (4%) had the same R_f as 6β--hydroxy-Δ^1-THC. However, due to the low amount of this substance its identification as 6β-hydroxy-Δ^1-THC was not definitive.

No conversion of Δ^1-THC to monohydroxylated compounds could be detected when an *in vitro* experiment was carried out with brain tissue. This is in agreement with earlier findings [42] that mouse brain is incapable of metabolizing Δ^1-THC. It appears that any metabolites of Δ^1-THC found in the CNS *in vivo* must be formed at a site remote from the brain and transported to it in the blood stream. However, Burstein *et al.* [unpublished results] have found the monkey brain to be able to metabolize Δ^1-THC.

The relative proportions of the 6α- and 7-hydroxy metabolites found *in vivo* in mouse brain were in good agreement with those observed *in vitro* using a 10,000g supernatant of the liver (see p.12). In addition to these two metabolites Δ^1-THC-6-one was identified as an *in vitro* metabolic product. It has been reported to be pharmacologically inactive in the rhesus monkey [44].

The mean molar ratios of brain concentrations of unchanged Δ^1-THC to total monohydroxylated metabolites in three separate experiments were found to be 7:1, 9:1, and 8:1 at 20 min after i.v. administration of Δ^1-THC. Corresponding brain ratios of about 7:1 and 10:1 have been obtained in rats at 15 min [51] and in squirrel monkeys at 30 min [52] after i.v. administration of 5 and 10 mg/kg Δ^1-THC, respectively. Previous work [53] correlating mouse brain levels of cannabinoids with their cataleptic effect yielded a molar potency of 7.1 for 7-hydroxy-Δ^1-THC relative to Δ^1-THC. Christensen *et al.* [42] found that 7-hydroxy--Δ^1-THC was twice as potent as the parent compound when injected

i.v. to mice. 6α-Hydroxy-Δ^1-THC, when administered i.v. to mice, has been variously reported to have 1/6 of the activity of Δ^1--THC [54] and to be "much less active" than 6β-hydroxy-Δ^1-THC (itself 1/4-1/2 as active as Δ^1-THC) [55]. From these observations it seems likely that 6α-hydroxy-Δ^1-THC is less potent than 7-hydroxy-Δ^1-THC in the mouse brain.

From what is now known of relative potencies and concentrations of cannabinoids in mouse brain, the major part of the cataleptic effect of i.v. administered Δ^1-THC in mice would seem to be attributable to Δ^1-THC.

Metabolites of Δ^1-THC in bile

The *in vivo* metabolism of Δ^1-THC has been elucidated only to a limited extent. *In vitro* metabolites of Δ^1-THC will probably be formed as intermediates *in vivo*. However, only a few of the *in vitro* metabolites have been identified beyond doubt in blood and urine of man or experimental animals. The major *in vitro* metabolite, 7-hydroxy-Δ^1-THC, has been identified in human blood [55] and urine [56]. Evidence has been presented for the occurrence in human blood plasma of 6α,7-dihydroxy-Δ^1--THC together with two other compounds tentatively identified as 6α- and 6β-hydroxy- Δ^1-THC [55]. Most urinary metabolites seem to be carboxylic acids and conjugates. Hitherto, only a few acidic conversion products of Δ^1-THC have been identified from the urine [*cf.* 2,57]. The *in vivo* metabolite patterns of Δ^1-THC seem indeed to be very complex [*cf.* 58].

In paper IV the metabolite pattern of Δ^1-THC in the bile of rat is partly characterized. The rat was used since it is known to excrete Δ^1-THC mainly via the faeces [29,59] which is also responsible for the predominant excretion of Δ^1-THC in man [60].

Male rats were anesthetized with pentobarital, and the bile ducts were cannulated. Δ^1-THC-^{3}H in aqueous ethanol was injected,

and bile was collected up to 6 hr after administration. The elimination of Δ^1-THC and its metabolites by the liver into the bile was most rapid during the first hour after injection, and within 2-3 hr 50% of the dose was excreted (Fig. 5).

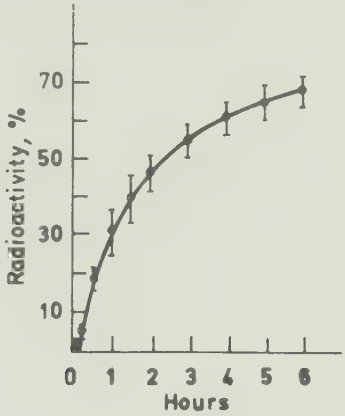


Fig. 5 Cumulative excretion of radioactivity in the bile after i.v. administration of Δ^1-THC-^{3}H to rats (1 mg/kg). Mean values ±SD are given from five rats.

Pronounced biliary excretion of metabolites of Δ^1-THC in rat has also been observed by others [61,62]. Previous reports have shown that the main route of elimination in the rat is via the faeces and that excretion is slow with some 60% eliminated in four days [62], or five days [29] and about 50% in one week [59]. These data indicate a pronounced enterohepatic circulation of THC metabolites in the rat which might be of considerable toxicological importance.

The predominant metabolites of Δ^1-THC in the bile were found to be acids (23%) and water-soluble conjugates (59%). However, small amounts of unchanged Δ^1-THC (0.1%) and CBN (0.05%) were present. They were identified by mass fragmentography and TLC by comparison with authentic samples. We have assumed CBN to be a metabolic product of Δ^1-THC formed by dehydrogenation and/or dehydration of oxygenated intermediates. Recently, McCallum [63]

has suggested that CBN, present in human plasma, was a metabolite of Δ^1-THC.

Nonconjugated 7-hydroxy-Δ^1-THC could not be identified in bile in spite of the fact that it is a major *in vitro* metabolite of Δ^1-THC in the rat [34]. As judged by mass fragmentography and polarity on TLC and Florisil column, probably only two mono--oxygenated metabolites of Δ^1-THC were eliminated as free compounds.

The elution pattern on Sephadex LH-20 of the acids (ether extract at pH 3, esterified) revealed at least three major compounds before the change to the more polar eluent. No more than 0.1%, if any of the total excreted radioactivity, could be due to Δ^1-THC-7-oic acid as evidenced by TLC with synthetic Δ^6-THC--7-oic acid as a reference.

The remaining aqueous phase (water-soluble conjugates) was submitted to hydrolysis by enzymes and alkali. No conjugates seemed to be present as sulphates*. Acidic aglycones released by glucuronidase showed a similar elution pattern on Sephadex as the free acidic metabolites.

Neutral aglycones released by β-glucuronidase and alkali were purified and separated on Florisil followed by Sephadex LH-20. According to mass fragmentography and TLC at least four mono-oxygenated aglycones were present.

One aglycone had an elution volume of 85 ml on Sephadex LH-20. It was revealed by mass fragmentography to be a side--chain hydroxylated compound, but the position of the hydroxyl group could not be determined until we had the reference 3"--hydroxy-Δ^1-THC. On the basis of MS, GLC and TLC data, the bile metabolite was found to be identical to 3"-hydroxy-Δ^1-THC (iso-

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*The designation as sulphate or glucuronide is based solely on the sensitivity of conjugates to corresponding enzymatic cleavage and their water solubility.

lated from lung).

Another aglycone of glucuronic acid showed an elution volume of 135 ml on Sephadex. It was found to be 7-hydroxy-Δ^1-THC by mass fragmentography and TLC. 7-Hydroxy-Δ^1-THC, on the basis of TLC, was reported to occur in a free form in the bile [62], but we were only able to identify it after hydrolysis of conjugates.

Thus, unchanged Δ^1-THC and CBN were eliminated in low amounts (0.05-0.1%) in the bile. Free mono-oxygenated metabolites of Δ^1-THC were present in only a few per cent. None were identical with 7-hydroxy-Δ^1-THC or 6β-hydroxy-Δ^1-THC. The main part of the non-conjugated metabolites were present as compounds more polar than 7-hydroxy-Δ^1-THC and as carboxylic acids.

About 60% of the metabolites were eliminated as water-soluble conjugates. Hydrolysis with glucuronidase liberated aglycones which were mainly neutral. Two of these metabolites were identified as 7-hydroxy-Δ^1-THC and 3"-hydroxy-Δ^1-THC.

Biotransformation of other cannabinoids

Since Δ^1-THC and Δ^6-THC have been established as the psychotomimetically active principles of cannabis, the research has mainly been focused on these compounds. As mentioned earlier there are many other constituents present in cannabis, in particular CBN (3) and CBD (4). Evidence exists that these two compounds may interfere with the actions of THC.

CBN present in large quantities in cannabis has been suggested to potentiate the effects of Δ^1-THC [64,65]. An antagonism by CBN of the THC prolongation of barbiturate-sleeping time has been observed [66,67]. Recently, it was reported that high doses of CBN administered i.v. to man produced some psychoactive effects [68].

CBD which is demonstrated to be devoid of hallucinogenic

effect [*e.g.* 68,69] has been shown to interact with Δ^1-THC on the duration of pentobarbitone anaesthesia in animals [67,70] and to antagonize several exitatory effects of Δ^1-THC in man [71]. From recent evidence it is probable that CBD interacts with Δ^1-THC by inhibiting the metabolism of Δ^1-THC and also its metabolite 7-hydroxy-Δ^1-THC [72]. Likewise, CBD has been reported to possess anticonvulsant activity in the rat [73,74].

Since the interactions between the two cannabinoids and THC seem to be of importance for the overall effects of cannabis, we considered it worthwhile to study their metabolism (paper V-VII).

In vitro metabolism of CBN by the liver

When CBN-^{14}C was incubated with the microsomal fraction of rat liver, a major metabolite amounting to 83% of the converted material was produced (paper V). The structure of the metabolite was determined by MS and NMR data along the lines described previously for the characterization of 7-hydroxy-Δ^1-THC (paper I). The evidence obtained was consistent with the structure of 7-hydroxy-CBN (24; Fig. 6).

After paper V was published, synthetically prepared 7-hydroxy-CBN became available to us [75]. The metabolite was found to have the same GLC behaviour and identical NMR and MS as the synthetically prepared 7-hydroxy-CBN.

Independently, Wall [39] reported on the formation of 7-hydroxy-CBN by rat liver homogenate. Wall also found two minor metabolites that he tentatively identified as 2"-hydroxy-CBN (26) and 2",7-dihydroxy-CBN (27; Fig. 6).

When CBN-^{14}C was incubated with a rabbit liver homogenate, several metabolites were formed (paper VI). The major hydroxylated metabolite (44% of converted material) was 7-hydroxy-CBN (24) as revealed by MS, GLC and TLC comparison with a reference.

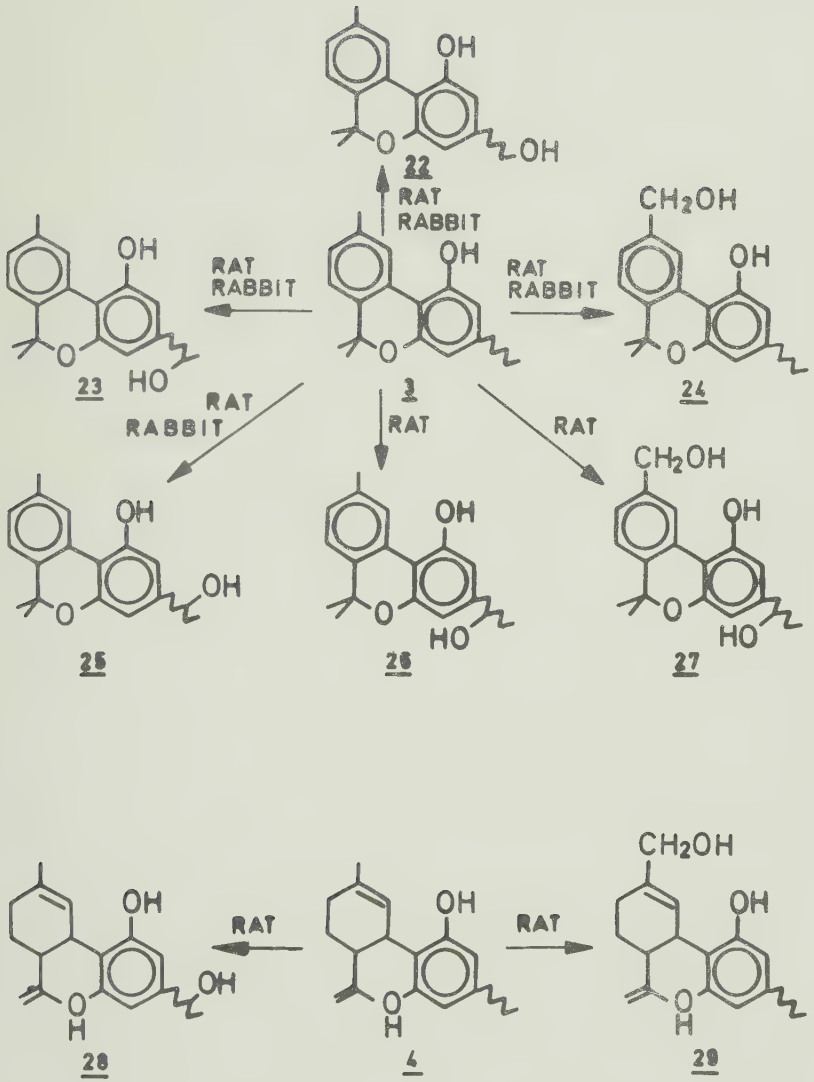

Fig. 6 Metabolic pathways for CBN (3) and CBD (4) using liver incubations.

Three metabolites were found to be hydroxylated in the pentyl side-chain (25,23,22; Fig 6). The mass spectra of these substances showed the presence of the fragments m/e 238 ($M^+-CH_3-C_4H_8OH$) and m/e 239 ($M^+-CH_3-C_4H_8O$) thus revealing hydroxylation in the side-chain in either 2"-, 3"-,4"- or 5"-position [*cf.*47]. Therefore, 2"-, 3"- and 5"-hydroxy-CBN were synthesized for comparative purposes. The MS, GLC and TLC data of 3"-hydroxy-CBN and 5"-hydroxy-CBN were identical with the corresponding data of metabolites 25 and 22, respectively. 3"-Hydroxy-CBN accounted for only 1% while 5"-hydroxy-CBN amounted to 5% of the converted material.

The third side-chain hydroxylated metabolite (30%) showed a TLC mobility between 3"- and 5"-hydroxy-CBN. The mass spectrum of the silylated metabolite showed the fragment m/e 117 (α-cleavage between C-3" and C-4") indicating hydroxylation in the 4"-position [*cf.*48]. The NMR data [*cf.* 48] also showed hydroxylation in 4"--position. A characteristic feature was the absence of the triplet signal corresponding to the protons on C-5" usually centered at δ=0.9 ppm. Instead, a doublet at δ=1.21 ppm was present. Thus, from MS and NMR the metabolite was given the structure of 4"--hydroxy-CBN.

Earlier findings by Wall [39] which showed that the rat could produce side-chain hydroxylated compounds were confirmed by us (paper VI). In addition to 2"-hydroxy-CBN, tentatively identified by Wall [39], we found 3"-,4"- and 5"-hydroxy-CBN when CBN-^{3}H was converted by an enriched microsomal fraction of rat liver. The side-chain hydroxylated metabolites were present as minor compounds, 2% of converted material, while 7-hydroxy-CBN made up for about 70%.

A species difference between rat and rabbit was thus observed in the metabolism of CBN.

In vitro metabolism of CBD by the liver

In paper VII the metabolism of CBD-^{14}C by the rat liver is described. CBD was converted under the same conditions as was used for CBN-^{14}C in paper V. Several metabolites were formed which could be separated by GLC. NMR and MS revealed the major hydroxylated metabolite to be 7-hydroxy-CBD (29; Fig. 6).

A second metabolite isolated was found by MS to be hydroxylated in the pentyl side-chain. The mass spectrum of this product showed the presence of the fragment m/e 247 but lacked the fragment m/e 231 which indicated hydroxylation in the side-chain [*cf.* 47]. Therefore 1"-,2"- and 3"-hydroxy-CBD were synthesized for comparative studies. The MS, GLC and TLC data of 3"-hydroxy-CBD were indistinguishable from the corresponding data of the metabolite. Thus, the hydroxyl group in the pentyl side-chain was determined to be in the 3"-position (28; Fig. 6).

The *in vitro* experiments described above have demonstrated that the initial hydroxylation of THC, CBN and CBD occur on the C-7 methyl group or on a carbon allylic to the double bond in the terpene moiety. Hydroxylation has also been found on various carbons in the aliphatic pentyl side-chain. Thus, the metabolism of CBN and CBD has been found to be analogous to that of THC.

Protein binding of Δ^1-THC and 7-hydroxy-Δ^1-THC

The binding of a drug to plasma protein, which usually is a reversible process, influences the behaviour of the drug in the body. The drug-protein complex is of high molecular weight and as such unavailable for membrane transport. The binding can influence the distribution of the drug and thereby its access to the active sites. It might inhibit the removal of the drug from the body by restricting the availability of the substrate to

metabolic and excretory organs.

Metabolic studies on Δ^1-THC indicated that the drug might be bound to proteins. This initiated our experiments on the interactions of Δ^1-THC and its metabolite 7-hydroxy-Δ^1-THC with proteins. In paper VIII the bindings of Δ^1-THC to human plasma proteins is described. Paper IX deals with the binding of 7-hydroxy-Δ^1-THC to human plasma proteins while paper X shows the distribution of both drugs in whole blood of man.

Binding of Δ^1-THC to plasma proteins

Initially, we tried to determine the binding properties of Δ^1-THC with an ultrafiltration method. The data obtained clearly indicated that Δ^1-THC to a large extent was bound to plasma proteins, but the quantitative significance of the results was questionable due to the low solubility of Δ^1-THC in buffer solution. Instead, we developed different electrophoretic techniques (paper VIII).

Δ^1-THC was incubated with human plasma and subjected to disc electrophoresis [76,77]. Distribution of radioactivity and proteins in the gel was determined. The radioactivity was concentrated mainly in two peaks. The major peak was located in the albumin area and a minor one around transferrin. Altogether, they accounted for 81-87% of the total activity present in the gel.

To be able to further correlate the protein bound Δ^1-THC with certain plasma protein fractions, agarose and agar gel electrophoresis were used. The agarose gel electrophoresis [78] showed that the major radioactive peak moved slightly less than the albumin band (Fig. 7). Immuno-electrophoresis of the agarose gel showed the radioactive peaks to coincide with the location of lipoprotein bands.

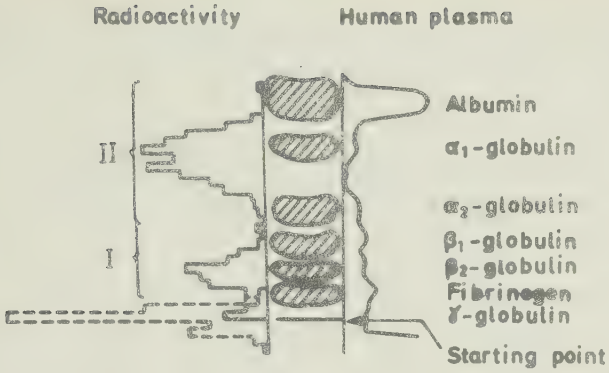

Fig. 7 Agarose gel electrophoresis of Δ^1-THC-^{3}H incubated with human plasma (—) and with phosphate buffer (- - -). To the right is shown the evaluation of colour density of the gel after staining of the proteins.

Agar gel electrophoresis of human plasma incubated with Δ^1-THC-^{3}H was performed. Determination of radioactivity was carried out by radiochromatogram scanning of the gel prior to its staining for lipoproteins. The major part of the radioactivity was connected with two lipoprotein areas.

Incubation of Δ^1-THC-^{3}H with purified human albumin revealed that there was no radioactivity associated with the albumin band after electrophoretic separation.

Thus, we found Δ^1-THC to be bound to 80-95% to human plasma proteins of which the lipoproteins seemed to be the main carriers. Our results have later been supported by the results of Klausner *et al.* [79] who used ultracentrifugation, to find 60-70% of Δ^1-THC bound to lipoproteins. Recently, a similar protein binding pattern of Δ^1-THC in rat serum has been observed by Fehr and Kalant [80] using agarose gel electrophoresis.

Binding of 7-hydroxy-Δ^1-THC to plasma proteins

The extent of binding of 7-hydroxy-Δ^1-THC-^{14}C to human plasma proteins was determined by equilibrium dialysis [81] and ultrafiltration. By dialysis 7-hydroxy-Δ^1-THC was bound 96-99%

and by ultrafiltration 94%. Equilibrium dialysis of 7-hydroxy--Δ^1-THC in a 4.5% solution of purified human albumin showed a protein binding of 82-83%.

Electrophoresis in agarose and in polyacrylamide were carried out to determine which proteins 7-hydroxy-Δ^1-THC interacted with.

Agarose gel electrophoresis of 7-hydroxy-Δ^1-THC-^{14}C incubated with human plasma showed a broad peak in the albumin--α_1-globulin area, which contained 73-80% of the total radioactivity present in the gel. A small peak (6-10%) coincided with the β-lipoprotein band. Incubation of the drug with human albumin and subsequent electrophoresis of the incubate showed an albumin binding of 80-85% which was in agreement with the dialysis experiments. Thus, albumin was found to interact with 7-hydroxy-Δ^1-THC. However, since the agarose gel could not separate the albumin band clearly from *e.g.* α_1-lipoprotein, it was difficult to estimate the radioactivity associated with the albumin.

With the use of a polyacrylamide gel of graded porosity [82] albumin could be better separated from other proteins, and the radioactivity in this band was determined (Fig. 8) to be about

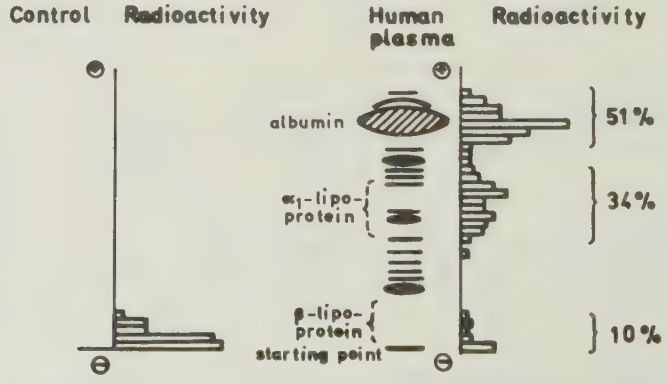

Fig. 8 Electrophoresis in polyacrylamide gel of graded porosity of 7-hydroxy--Δ^1-THC incubated with plasma (sample) and buffer (control).

50% of the total. The broad radioactive peak in the middle of the gel contained some 35% of the total radioactivity present in the gel. The proteins located in this area have a molecular weight of 150,000-250,000 [82] *e.g.* α_1-lipoprotein. Indications were that 7-hydroxy-Δ^1-THC might be interacting with lipoproteins.

To verify the binding of 7-hydroxy-Δ^1-THC to the lipoproteins, ultracentrifugation was used. By ultracentrifugation the lipoproteins, on the basis of their densities, were separated into two fractions, the low density lipoprotein fraction (LDL; <1.063 g/ml) and the high density lipoprotein fraction (HDL; density 1.063-1.21 g/ml). Approximately 7% of the radioactivity in the plasma incubate was present in the LDL fraction which contained β-lipoprotein. About 20% of the radioactivity was found in the HDL fraction containing α_1-lipoprotein.

The results of the electrophoresis and ultracentrifugation experiments show that 7-hydroxy-Δ^1-THC is strongly bound to plasma proteins and that both albumin, α_1-lipoprotein and to a minor extent β-lipoprotein, are associated with 7-hydroxy-Δ^1-THC.

Fehr and Kalant [80] found, when studying the *in vivo* distribution of Δ^1-THC-^{14}C in rat serum, shortly after administration most of the radioactivity bound to the lipoproteins. At 15 min an increased concentration of radioactivity was observed in the albumin area. The high albumin binding was suggested to represent the binding of metabolites. The greater lipophilic character of Δ^1-THC as compared to its hydroxylated metabolite seem to reflect the binding properties of these compounds.

Binding of Δ^1-THC and 7-hydroxy-Δ^1-THC to whole blood

The binding of drugs is usually determined in plasma or purified serum albumin solutions. Few studies have been made on drug binding to blood cells which is a main constituent of circulating blood.

In paper X we have investigated the binding of (-)-Δ^1-THC and (-)-7-hydroxy-Δ^1-THC to both plasma proteins and blood cells in man. We also examined the binding properties of the enantiomorphic (+)-Δ^1-THC which does not occur naturally and has little pharmacological activity [83].

Equilibrium dialysis was used to determine the total binding of the drugs to human plasma proteins [81]. The compounds were dissolved in buffer and dialysed against human plasma at 37°C for 24 h. The results are presented in Table 1.

Binding of cannabinoids to blood cells was determined by incubation with whole blood and with "blood cell suspension" (washed blood cells suspended in buffer) followed by centrifugation. Whole blood and blood cell suspension were incubated with radiolabelled (-)-Δ^1-THC, (+)-Δ^1-THC or (-)-7-hydroxy-Δ^1-THC. At fixed intervals aliquots were withdrawn and centrifuged, and the radioactivity present in the supernatants, plasma and buffer, was determined by liquid scintillation counting. The drug concentration in whole blood and blood cell suspension was determined by solubilizing aliquots with Soluene-350 before counting for radioactivity. With these data available, together with the hematocrit values, the distribution of the drug to blood cells, plasma proteins and plasma water could be calculated [84]. The results are shown in Table 1.

Table 1

Distribution of (-)-Δ^1-THC, (-)-7-hydroxy-Δ^1-THC and (+)-Δ^1-THC in whole blood and blood cell suspension *in vitro*.

Compound	% bound in plasma	% in whole blood distributed to:			% in blood cell suspension distributed to blood cells
		plasma water	plasma proteins	blood cells	
(-)-Δ^1-THC	97	3	88	9	96
(-)-7-hydroxy-Δ^1-THC	99	1	90	9	98
(+)-Δ^1-THC	97	3	87	10	97

Since the results agreed closely only mean values of 2-3 experiments are given. Drug concentrations in plasma and blood: 0.3-0.5 µg/ml.

As evident from Table 1, all compounds are highly bound with a total binding of 97-99% to the plasma proteins. This is in agreement with our earlier findings in humans (paper IX) and with Garrett and Hunt [85] who have studied the binding of (-)-Δ^1-THC to dog plasma proteins. The distribution of the compounds in whole blood showed a binding to the red blood cells of only 9-10%. When the cannabinoids were incubated with washed red blood cells in the absence of plasma proteins, they showed an extensive binding to the cells. The binding to plasma proteins and blood cells seemed to be independent of concentration over a wide range.

It is generally assumed that only the proportion of a compound which is in equilibrium with the unbound fraction in plasma is pharmacologically active. Since the peak levels of Δ^1-THC in man appears to be 25-50 ng/ml [86] and the levels of 7-hydroxy-Δ^1--THC even lower [*e.g.* 60] the activity of the drug, on a weight basis, must be remarkable.

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